Synergistic Activation of RLD-1 by Agents Triggering PKA and PKC Dependent Signalling

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RLD-1 and OR-1 are closely related orphan nuclear receptors that can be activated by certain oxysterols. To obtain cells stably expressing RLD-1 or OR-1, CHOK1 cells were successively transfected with a DGRE2-ALP reporter and GR-RLD-1 or GR-OR-1 chimeric constructs. The selected cell clones that showed low background activity of the reporter and maximum fold induction by 22R(OH)cholesterol were used for subsequent experiments. Treatment of the cells with PGE₂, TPA, or 8-bromo-cAMP alone did not transactivate the reporter. However, the induction of the reporter by 22R(OH)cholesterol was markedly enhanced in the presence of PGE₂, TPA, 8-bromo-cAMP, or forskolin in cells expressing GR-RLD-1. The enhancement was inhibited by H-89 and bisindolylmaleimide, both inhibitors of protein kinases. These results suggest that transactivation by ligand-activated RLD-1 may be further modulated/regulated through other signal transduction pathways involving phosphorylation catalyzed by protein kinases. © 1998 Academic Press

The nuclear receptor LXR α /RLD-1(1, 2), and its close relative LXR β /OR-1(also described as UR/NER/RIP 15)(3-6) are orphan members of the nuclear receptor superfamily. They were shown to form heterodimers with RXR that bind to a DR-4 type responsive element, conferring constitutive transcriptional activation and/or enhanced response to the RXR ligand 9-cis retinoic acid (1-3, 5,6).

In an attempt to find potential activators, Janowski et al. identified a specific subset of oxysterols that activate LXR α in CV-1 cell cotransfection assays (7). Among them, 22R(OH)cholesterol showed the highest activation potential. Furthermore Lehmann et al.(8) identified an LXR response element in the rat choles-

terol 7 α -hydroxylase gene and additional activators of the oxysterol types, among which 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol were most potent. As these oxysterols can activate LXR α/β at physiological concentrations, the activation of LXR by oxysterols was proposed to be a new hormonal signaling pathway that may play a critical role in the regulation of cholesterol homeostasis, e.g. via LXR α acting as an important sensor of cholesterol metabolites.

Recognizing the potentially important role of RLD-1/OR-1 in the regulation of steroid metabolism, it is important to test the receptor activation capacity of a wider range of compounds as ligands for RLD-1/OR-1 and the putative influence of other signalling pathways. We therefore established cell clones stably expressing chimeric constructs of these receptors along with a suitable reporter gene construct. Upon screening of various compounds we observed that agents triggering other signal transduction pathways markedly enhanced the activation of RLD-1 by 22R(OH)cholesterol and that this enhancement could be abolished by the addition of protein kinase inhibitors such as H-89 and BIM.

MATERIALS AND METHODS

Plasmids. The chimeric receptor constructs were generated by replacing the natural DNA binding domain of OR-1 or RLD-1 with the glucocorticoid receptor DBD employing suitable restriction sites or PCR mutagenesis. The GR-OR-1 construct contains aa1-75 of OR-1 fused in frame to aa419-500 of the GR-DBD followed by aa163-446 of OR-1. The GR-RLD-1 construct contains aa1-93 of RLD-1 fused in frame to aa419-500 of GR-DBD followed by aa180-455 of RLD-1. The correct in frame fusions were confirmed by sequencing. Both constructs were subcloned in the pCMX vector yielding the pCMX-GR-OR-1 and the pCMV-GR-RLD-1 expression vectors, respectively.

The pMSG vector from Pharmacia was used for the construction of the reporter, DGRE2-ALP, where the major GRE sites in MMTV have been replaced by one single GRE cloned between a Cla I and Sac I site. The sequence of the inserted GRE is: (upper strand) 5'-CGAT AGAACA CAG TGTTC T GAGCT-' and (lower strand) 5'-C AGAACA CTG TGTTC TA-3'. The secretable form of ALP (human placental alkaline phosphatase cDNA) was cloned as described by

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Berger et al.(9) and inserted. The MMTV sequence 5' of the Sac I site has been deleted but the sequence between the Sac I site and the insertion comes from MMTV, i.e. the GRE $\frac{1}{2}$ site, the NF-1 and Oct-1 binding sites including the TATA binding region and part of the MMTV leader sequence is included in this construct.

Cell culture and stable transfection. CHO K1 cells were maintained in Ham's F-12 medium (SVA, Uppsala, Sweden) supplemented with 10% fetal calf serum (Gibco BRL). For stable transfections, cells were seeded at a density of 25,000 cells/cm² on 6 cm round plates in 5 mL medium and grown for 24 h. Transfections were performed with lipofectamine (Gibco BRL) according to the manufacturer's instructions. Each plate received 3 μ g of DGRE2-ALP and 0.3 μ g psV Neo. Transfected cells were selected in the presence of 0.8 mg/mL medium of G418 (10). The colonies grown were pooled to form a reporter clone mix. The expression of the DGRE2-ALP in this reporter clone mix was confirmed by the induction of the ALP enzyme by dexamethasone after transient transfection with pMT-GR (11). The reporter clone mix was further transfected with GR-RLD-1 or GR-OR-1 expression vectors (1 or 5 μ g/plate) together with pKSV Hygro (0.1 or 0.5 μ g/plate respectively) (12) and selected with 0.8 mM hygromycin. Fifty clones were picked from each plate, expanded and tested for their responsiveness to 5 μ M 22R(OH)cholesterol, the known activator of hLXR α . The clone that showed maximum fold induction of the reporter gene activity was used for the following experiments. The stable integration and expression of the GR-RLD-1 and GR-OR-1 constructs in the selected clones were confirmed by Northern, Southern and Western blot analysis (data not shown).

Activation experiments and ALP assay. Stably transfected cells were seeded at a density of 25,000 cells/well in 96 well plates in 0.1 mL medium containing 10% DCC (Dextran-coated charcoal treated) FCS and grown for 24 h. Cells were washed with Coon's F-12 medium (SVA, Uppsala, Sweden) prior to the addition of 0.1 mL of activation medium. Stock solutions of 22R(OH)cholesterol, forskolin and TPA in DMSO, PGE₂ in ethanol and 8-bromo-cAMP in water were diluted to the desired concentrations with Coon's F-12 medium containing 5% serum replacement (TCS Biological Ltd., UK). Basal levels of expression of reporter activity were obtained with cells treated with medium containing vehicles (DMSO or ethanol) at a concentration equal to those used for maximal concentrations of activator/synergistic effectors. Cells were incubated with these activation media at 37°C for 48 h. Culture supernatants were collected and assayed for alkaline phosphatase activity using CSPD (Tropix) as a substrate and Sapphire as an enhancer. Chemiluminescence was measured in a luminometer (Labsystems Luminoscan). The toxicity of the treatment of cells were checked by the MTS assay (SDS, Falkenberg, Sweden). PGE₂ is purchased from Cayman Chemicals; forskolin, TPA, 8-bromo-cAMP, H-89 and bisindolylmaleimide I were from Calbiochem Co.

RESULTS

Prior to the stable transfection procedure, the induction of GR-RLD-1 and GR-OR-1 by 22R(OH)cholesterol on the DGRE2-ALP reporter was confirmed in transient co-transfection experiments (data not shown). The dose-response curve of three selected clones to 22R(OH)cholesterol is shown in Fig. 1. Cells stably expressing the GR-RLD-1 chimera were more responsive to 22R(OH)cholesterol than cells expressing the GR-OR-1 chimera that was only very moderately induced (1.5 fold induction at 5 μ M). The maximum induction of the GR-RLD-1 clone was achieved at the concentration of 25 μ M 22R(OH)cholesterol resulting in a 12.5

FIG. 1. Dose response curve of 22R(OH)cholesterol in CHOK1 cells stably expressing the GR-RLD-1 receptor chimera and/or the DGRE2-ALP reporter. CHOK1 cells were successively transfected with the reporter and the GR-RLD-1 or GR-OR-1 receptor construct. Clones showing low background and maximum fold induction of the reporter by 5 μ M 22R(OH)cholesterol were expanded and treated with various concentrations of 22R(OH)cholesterol for 48 h. The cultural supernatant was analyzed for alkaline phosphatase reporter activity. Data shown are direct reading values from the luminometer and are means $\pm$ S.D. of triplicates from one experiment and are representative of at least three experiments.

fold induction of the reporter gene activity. Induction with 50 μ M 22R(OH)cholesterol resulted in a decrease of activity due to the toxicity of this compound to cells at 50 μ M or higher concentrations. The EC₅₀ of the dose-response curve for the GR-RLD-1 clone was 5.38 μ M. The reporter cell line was not affected by 22R(OH)cholesterol at all concentrations tested.

To test the possibility that these receptors are activated not only by their cognate ligand but also by other signalling pathways we tested several compounds known to act as second messengers and/or to activate protein kinases. Treatment of the cells stably expressing GR-RLD-1 with various concentrations of PGE₂ alone did not induce reporter gene activity (Fig. 2A). However, when these cells were treated with increasingly amounts of PGE₂ in the presence of 5 μ M 22R(OH)cholesterol, the basal level of induction by 5 μ M of 22R(OH)cholesterol alone was raised with increasing concentrations of PGE₂ to more than 3-fold (Fig. 2A). Similar treatment of the cells with increasing concentrations of 22R(OH)cholesterol in the presence of 50 μ M PGE₂ markedly increased the induction of the reporter gene activity compared to those induced by 22R(OH)cholesterol alone (Fig. 3). No enhancing effects of PGE₂ were seen when the concentrations of 22R

FIG. 2. PGE₂, TPA, forskolin, and 8-bromo-cAMP enhance the transactivation of GR-RLD-1 by 22R(OH)cholesterol in a concentration dependent manner. CHOK1 cells stably expressing the GR-RLD-1 receptor chimera and the DGRE2-ALP reporter were treated with various concentrations of PGE₂ (A), TPA (B), forskolin (C), or 8-bromo-cAMP (D) in the absence or presence of 5 μ M 22R(OH)cholesterol for 48 h and the cultural supernatant was analyzed for alkaline phosphatase reporter activity. Data shown are direct reading values from the luminometer and are means $\pm$ S.D. of triplicates from one experiment and are representative of at least two experiments.

(OH)cholesterol were too low to be active, i.e., lower than 1 μ M (Fig. 3).

As PGE₂ is known to act through PKA or PKC dependent signalling mediated by membrane receptors (13) we decided to test other agents triggering these pathways. In a way similar to PGE₂, although at much lower concentrations, TPA, a phorbol ester that activates protein kinase C, enhanced the activity of the reporter in the presence of RLD-1 up to 4-5 fold compared to 22R(OH)cholesterol alone (Figs. 2B and 3). In a corresponding experiment with increasing amounts of 22R(OH)cholesterol in the presence of a fixed concentration of 10 nM TPA, an up to 4-fold induction was achieved (Fig. 3). Forskolin, an activator of adenylate cyclase, could enhance the activation to an even higher extent, i.e. up to 15 fold at 50-100

μ M concentrations (Fig. 2C). The cAMP analogue 8-bromo-cAMP also showed an enhancing effect but at much higher concentrations (Fig. 2D). The synergistic effects of these agents were not observed with the reporter clone mix or cell clones stably expressing GR- OR-1(Figs. 4 and 5).

The agents tested are known to activate protein kinase A and/or protein kinase C. To investigate if the synergistic effect observed is related to phosphorylation events catalysed by these protein kinases, inhibitors of these enzymes were added to cultured cells treated with 22R(OH)cholesterol and the various effectors. The synergistic activation by PGE₂ and TPA was completely inhibited by 6 μ M of Bisindolylmaleimide I (BIM)(Fig. 5 A,B). However, this concentration of BIM only partially inhibited the synergistic effects

FIG. 3. The transactivation of RLD-1 by various concentrations of 22R(OH)cholesterol is enhanced by PGE₂ and TPA. CHOK1 cells stably expressing the GR-RLD-1 receptor chimera and the DGRE2-ALP reporter were treated with various concentrations of 22R(OH)cholesterol in the absence or presence of 10 nM TPA or 50 μM PGE₂ for 48 h and the cultural supernatant was analyzed for alkaline phosphatase reporter activity. Data shown are direct reading values from the luminometer and are means ± S.D. of triplicates from one experiment and are representative of at least three experiments.

of forskolin and 8-bromo-cAMP. On the other hand, H-89 inhibited the synergistic effects of PGE₂ and Forskolin to almost 100%, in contrast to the less complete inhibition on the enhancing effects of TPA and 8-bromo-cAMP (Fig. 4).

DISCUSSION

In this study, we established cell clones according to the inducibility of reporter gene activity by 22R(OH)cholesterol, a known activator of LXRs. The EC₅₀ value of 22R(OH)cholesterol activation of GR- RLD-1 found in this study was 5.4 μM and is comparable to the results from previous transient-transfection studies varying from 1.5 μM (Janowski et al., 1996) to more than 10 μM as reported by Lehmann et al. (1997). However, cell clones stably expressing GR- OR-1 chimera responded to 22R(OH)cholesterol very poorly which is in contrast to data presented by Lehmann et al. (1997) where hLXRβ, the human analogue of rat OR-1, was activated to a greater extent by 22R(OH)cholesterol than was hLXRα (RLD-1). One possible explanation could be that the GR-OR-1 construct used in this study includes the N-terminus of OR-1, a part of the protein

that is not present in the LBD fusions used by other groups. The PEST sequences in the OR-1 N-terminus might render the receptor protein more susceptible for proteolysis and thus result in a shorter-half life.

The synergistic effects of PGE₂, TPA, forskolin and 8-bromo-cAMP on the activation of RLD-1 by 22R(OH)cholesterol are evident from the results of induction experiments that either fixed the concentration of the activator and varied the concentration of the synergistic effector or vice versa. With the exception of forskolin, the three remaining effectors alone did not activate the receptors.

Forskolin may activate the adenylate cyclase pathway resulting in elevated cAMP levels which in turn may lead to elevated PKA activity. Treatment of cells with forskolin alone not only induced reporter gene activity in the cell clone expressing GR- RLD-1, but also activated the cell clone expressing GR- OR-1 and the reporter clone mix to an extent comparable to or even greater than 22R(OH)cholesterol (Fig. 4C). This induction was abolished almost completely by H-89, which is a PKA specific inhibitor at the concentration used. Moreover, 1,9-dideoxy-forskolin, a structural analogue of forskolin that does not activate adenylate cyclase, did not activate these cell clones (data not shown). As 1,9-dideoxy-forskolin did not enhance the activation of GR- RLD-1 by 22R(OH)cholesterol, the cAMP dependent pathway seems to be essential for the enhanced activation by forskolin. This is further supported by the almost complete elimination of the effect of forskolin by the addition of 6 μM of H-89. Since the K_i values of H-89 for PKA and PKC are 48 nM and 31.7 μM, respectively, PKA is presumably almost completely inhibited at a 6 μM concentration of H-89 while PKC is only partially affected. Furthermore, the forskolin mediated effect was only partially abolished by the addition of 2 μM BIM. The K_i values of BIM for the inhibition of PKA and PKC are 2 μM and 10 nM, respectively. With 2 μM of BIM, therefore, PKC is presumably almost completely inhibited while PKA is only partially affected.

As expected, the enhancement mediated by TPA, a PKC activator, was only partially abolished by 6 μM of H-89 in contrast to the almost complete inhibition when BIM was added to the medium. Considering the low concentrations used (10 nM), TPA showed the strongest effect on reporter gene activation in the GR- RLD-1 expressing cells. Interestingly, RLD-1 contains a putative recognition sequence for PKC (RXXSXR) in the hinge region of the protein at aa225-230 (RSFSDR) in contrast to OR-1 that does not contain a similar site. Cells expressing this chimera were not affected by TPA treatment.

The enhancing effect of PGE₂ on the activation of GR- RLD-1 was almost completely inhibited by both the 6 μM H-89 and 2 μM BIM treatment. The effects

FIG. 4. H-89 inhibits the synergistic effects of PGE₂, TPA, forskolin, and 8-bromo-cAMP on transactivation of RLD-1 by 22R(OH)cholesterol. CHOK1 cells stably expressing the GR-RLD-1 or the GR-OR-1 receptor chimera and the DGRE2-ALP reporter or the reporter alone were treated with 5 μ M 22R(OH)cholesterol alone, 50 μ M PGE₂ (A) alone, 10 nM TPA (B) alone, 50 μ M forskolin (C) alone, or 125 μ M 8-bromo-cAMP (D) alone or in combinations in the presence or absence of 6 μ M H-89. The cultural supernatant was collected after 48 h and analyzed for alkaline phosphatase reporter activity. Data shown are direct reading values from the luminometer and are means $\pm$ S.D. of triplicates from one experiment and are representative of two experiments.

of PGE₂ on cells and tissues are diverse and sometimes even opposite. This diversity is due to the various PGE₂ receptor subtypes (EPs) being coupled to a variety of signal transduction pathways, including PKA and PKC (13). It is not clear if one or more EP subtypes are expressed in the CHO K1 cells used in this study.

The results of this study imply that the transactivation of LXR α /RLD-1 may be further modulated/regulated by mechanisms involved in signal transduction pathways, such as phosphorylation associated with protein kinases. The modulation/regulation of the transcription activity by phosphorylation has been demonstrated in other nuclear receptors previously. For example, the orphan nuclear receptor steroidogenic factor-1 (SF-1) has been shown to regulate the cAMP-mediated transcription of cytochrome P450 steroid hydroxylase genes (14) and to be activated synergistically by phorbol esters (15). The human estrogen receptor

can be activated through phosphorylation by a mitogen-activated protein kinase (16). Phosphorylation of thyroid hormone receptor (TR β 1) resulted in enhanced dimerization (17) with RXR and tissue specific stabilization (18). Phosphorylation of the MAPK site in the A/B region of PPAR γ inhibited both ligand-independent and ligand-dependent transactivation functions (19). Phosphorylation of Ser-350 of the orphan nuclear receptor NGFI-B resulted in a decrease in binding to the NGFI-B response element (20, 21).

The oxysterols that activate LXR seem to occur at the rate-limiting steps of cholesterol metabolism such as: steroid hormone biosynthesis, bile acid biosynthesis and conversion of lanosterol to cholesterol. LXR α is specifically expressed in tissues that are metabolically active, such as liver and adrenal gland. It is therefore possible that activation of LXR α by oxysterols may lead to transcriptional regulation of crucial metabolic path-

FIG. 5. Bisindolylmaleimide inhibits the synergistic effects of PGE₂, TPA, forskolin, and 8-bromo-cAMP on the transactivation of RLD-1 by 22R(OH)cholesterol. CHOK1 cells stably expressing the GR-RLD-1 or the GR-OR-1 receptor chimera and the DGRE2-ALP reporter or the reporter alone were treated with 5 μ M 22R(OH)cholesterol alone, 50 μ M PGE₂ (A) alone, 10 nM TPA (B) alone, 50 μ M forskolin (C) alone, or 125 μ M 8-bromo-cAMP (D) alone or in combinations in the presence or absence of 2 μ M bisindolylmaleimide. The cultural supernatant was collected after 48 h and analyzed for alkaline phosphatase reporter activity. Data shown are direct reading values from the luminometer and are means $\pm$ S.D. of triplicates from one experiment and are representative of at least two experiments.

ways (7). The further enhancement of 22R(OH)cholesterol triggered trans-activation of RLD-1 by agents stimulating PKA and/or PKC dependent signalling observed in this study implies that the transcriptional regulation of cholesterol metabolism by activator/ligand/metabolite may be further controlled by extracellular growth factors/regulators that transduce the signals via membrane receptor-mediated pathways. Interestingly, expression of cholesterol 7 α -hydroxylase gene expression was modulated not only by hormones but also by signal transduction pathways (22).

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